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ORIGINAL ARTICLE

Anti-tumor efficacy of Cloretazine (VNP40101M) alone and in combination with fludarabine in murine tumor and human xenograft tumor models

Li-mou Zheng · Zujin Li · Lanzhen Liu · Bai Louis Song · Ivan King

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Abstract Cloretazine (VNP40101M), a new sulfonylhydrazine alkylating agent, has demonstrated broad-spectrum anti-tumor activity in preclinical studies. In this study, Cloretazine was evaluated both as a monotherapy and in combination with fludarabine in murine tumor and human tumor xenograft models. Cloretazine significantly inhibited the growth of subcutaneously implanted tumors, including B16F10 murine melanoma in C57BL/6 mice, and H460 human lung carcinoma and WiDr human colon carcinoma in athymic nude CD1 mice. The inhibition of tumor growth by Cloretazine was dose dependent, increasing from 42.2 to 87% as the dose escalated from 100 to 150 mg/kg. Cloretazine showed equivalent efficacy but lower toxicity compared to cyclophosphamide in these models. The combination therapy, consisting of a single dose of 10 mg/kg Cloretazine plus five doses of 70 mg/kg fludarabine, given every other day intraperitoneally, significantly increased the long-term survival of BDF1 mice bearing the L1210 murine leukemia. On Day 65 post-tumor implantation, the combination therapy yielded a 90% survival rate compared to 40% for Cloretazine alone and 0% for fludarabine alone.

Keywords Cloretazine · VNP40101M · Fludarabine · Anti-tumor effect · Melanoma · Carcinoma · Leukemia

L.-m. Zheng (✉) · L. Liu · B. L. Song · I. King
Vion Pharmaceutical, Inc., 4 Science Park,
New Haven, CT 06511, USA
e-mail: lzheng@vionpharm.com

Z. Li
Department of Pathology,
Memorial Sloan-Kettering Cancer Center,
New York, NY, USA

Introduction

Deoxyribonucleic acid (DNA) alkylating agents are among the most effective chemotherapeutic agents currently available, but they are also highly toxic [14]. Although alkylating agents are considered a single class of therapeutic agents, they are heterogeneous in their chemical specificity for DNA rather than other cellular components and in their specific mechanisms of action for damaging DNA. Different types of DNA damage are repaired in different ways, resulting in different consequences for the cell. For example, alkylating agents may cause DNA crosslinks or single-strand DNA nicks, resulting in cell cycle arrest, DNA repair, and/or apoptosis (programmed cell death), depending on the type of DNA damage. These differences distinguish alkylating agents from each other with respect to their potency, safety profiles, and relative susceptibility to compensatory tumor resistance mechanisms.

The CENU (chloroethylnitrosourea) series of alkylating agents has long been used clinically to treat brain tumors, colon cancer, and lymphomas [6, 17]. Its clinical usefulness is limited, however, due to delayed and cumulative bone marrow depression and hepatotoxicity [10, 19]. Therefore, newer alkylating agents with potentially improved therapeutic properties have been developed.

Cloretazine (VNP40101M or 1,2-bis(methylsulfonyl)-1-(2-chloroethyl)-2-(methylamino) carbonylhydrazine) is a novel sulfonylhydrazine alkylating agent and chemotherapeutic prodrug with possible advantages over other alkylating agents. Upon decomposition, Cloretazine yields 1,2-bis(methylsulfonyl)-1-(2-chloroethyl) hydrazine (90CE) and methyl isocyanate. 90CE

further fragments to yield methyl 2-chloroethyldiazosulfone, a relatively specific 0^6-guanine chloroethylator [1]. The O^6-(2-chloroethyl)guanine adduct rapidly cyclizes to form N^1,O^6-ethanoguanine which, if not repaired, rearranges to form an N^1 to N^3 G-C interstrand cross-link. Cloretazine yields more than twice the molar yield of DNA cross-links than the CNUs [21]. In contrast to what is seen with the chloroethylnitrosoureas, no significant N^7 alkylations are seen with Cloretazine as evidenced by the lack of single strand nicking. N^7 alkylation of the purine ring leads to depurination and sugar-phosphate chain hydrolysis, causing a single strand nick, a common lesion observed with chloroethylnitrosoureas that produce chloroethylisocyanate (i.e. BCNU) [3, 4, 20].

The methyl isocyanate released from Cloretazine may enhance the 0^6-guanine alkylation of 90CE via inhibition of AGT (0^6-alkylguanine-DNA alkyltransferase), the enzyme responsible for the repair of the lesions produced by Cloretazine [7, 21]. The AGT inhibition results in higher levels of 0^6-guanine alkylation, and consequently, more interstrand DNA crosslinks [2]. In contrast, the decomposition of CENU compounds leads to DNA alkylation and therapeutically irrelevant activities, which contribute to the toxicity of these compounds. For example, BCNU (1,3-bis(2-chloroethyl)-1-nitrosourea), but not Cloretazine, inhibits cellular glutathione reductase, which may contribute to pulmonary toxicity [24].

Cloretazine has broad-spectrum activity against various syngeneic and human xenograft tumors in murine models [7, 25]. It has demonstrated efficacy against P388 and L1210 murine leukemias, M109 lung carcinoma, C26 colon carcinoma, and U251 human glioblastoma in mice [7]. Cloretazine also caused complete regression of U251 glioblastoma in mice and eradicated L1210 leukemia cells implanted intracranially in mice, rivaling the efficacy of BCNU, an effective chemotherapeutic agent for the treatment of brain tumors [7]. Bio-distribution studies confirmed that Cloretazine or its metabolites crossed the blood brain barrier [16]. Furthermore, Cloretazine was active against L1210 leukemias that resist other alkylating agents, including BCNU, cyclophosphamide, or melphalan (Alkeran) [7]. Human clinical trials indicated that Cloretazine, alone or in combinations, could be used for the treatment of acute myeloid leukemia [27].

Mechanistically, DNA crosslinking that results from Cloretazine treatment induces excision repair processes, which increase the incorporation of nucleosides. Consequently, alkylating agents and nucleoside analogs act synergistically, increasing the incidence of apoptotic cell death relative to either class of agents alone [26]. Furthermore, since increased excision repair is a hallmark of acquired resistance to alkylating agents [9, 18], the combination should maintain drug efficacy due to the increased incorporation of nucleoside analogs or lower acquired resistance. Combination chemotherapy has long been used to treat leukemia [8] and other tumors [5], with the aim of increasing therapeutic benefits by overcoming drug resistance or by killing tumors with multiple mechanisms.

Fludarabine is a nucleoside analog that incorporates into DNA during replication and subsequently blocks DNA synthesis [13], leading to cell cycle arrest and apoptosis [11]. Fludarabine also directly inhibits enzymes involved in DNA replication [22]. Fludarabine has many clinical applications, and is the single most successful agent in chronic lymphocytic leukemia (CLL) clinical trials [15]. Combination therapies of fludarabine plus alkylating agents have been found to be more effective than fludarabine alone [12]; they are well tolerated in patients with CLL or non-Hodgkin's lymphoma [23].

In this study, inhibition of the growth of B16F10 murine melanoma, H460 human lung carcinoma, and WiDr human colon carcinoma implanted in mice following Cloretazine treatment was investigated. Cloretazine treatment effects in H460 and WiDr tumors in mice also were compared with cyclophosphamide. Finally, the long-term survival of the mice bearing L1210 leukemic cells was assessed after treatment with Cloretazine plus fludarabine.

Materials and methods

Cells and culture conditions

All cell lines were obtained from the American Type Culture Collection except B16F10 murine melanoma, which was provided by Dr. I. Fidler. B16F10 murine melanoma, H460 human lung carcinoma, and WiDr human colon carcinoma cell lines were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum; L1210 leukemia cell line was cultured in RPMI1640 with 10% fetal bovine serum. All cell lines were maintained at 37 ± 2°C in a humidified environment with 5% CO_2.

Animal care

Mice were obtained from Charles River Laboratories (Wilmington, MA, USA), and were handled, treated, fed, and housed according to USDA Animal Welfare Act (9 CFR Parts 1, 2, and 3) and Guide for the Care

and Use of Laboratory Animals (National Academy Press, Washington, DC, USA, 1996). The animals were housed in plastic cages with stainless steel covers containing five mice each. Autoclaved tap water was provided ad libitum in glass bottles, and mouse chow provided ad libitum in food hoppers. The animals were kept in a well-ventilated room maintained at 72 ± 2°F and provided with a 12-h light/12-h dark photocycle. Animals selected for these studies were as uniform as possible in age and weight; identification was made via ear tag. All mice were female, weighed approximately 20 g, and were 9 weeks of age (C57BL/6 and nude CD1 mice) or 10 weeks of age (BDF1). Body weight and tumor size were measured twice per week in all experiments, except for the L1210 leukemia model experiments, where body weight has measured once per week. The animals were observed daily for behavior and mortality, and euthanized in a chamber containing dry ice at the end of the study.

Tumor cell implantation

Upon passage of several generations in culture, log phase B16F10 murine melanoma, H460 human lung carcinoma, or WiDr human colon carcinoma cells were dislodged from flasks by trypsinization, washed, and reconstituted in 1 X PBS to a concentration of 2.5×10^6 cells/ml (B16F10 cells) or $2.5–5.0 \times 10^7$ cells/ml (H460 and WiDr cells). A total of 5×10^5 B16F10 cells in 0.2 ml was subcutaneously implanted into the right flank of C57BL/6 mice on Day 0 to generate the B16F10 murine melanoma model. A total of 5×10^6 H460 or WiDr cells in 0.2 ml was implanted into the right flank of nude CD1 mice to generate the H460 human lung or WiDr human colon carcinoma models. Cultured L1210 leukemia cells were washed and intraperitoneally (i.p.) implanted into BDF1 or nude CD1 stock mice, then passed for several generations in the stock mice. L1210 cell suspension was freshly harvested from the stock mice, and a total of 2×10^6 L1210 cells in 0.2 ml was injected i.p. into BDF1 mice. After implantations the mice were immediately grouped randomly in all experiments.

Treatment of mice with Cloretazine (VNP40101M)

Cloretazine (obtained from the Chemistry Department, Vion Pharmaceuticals, Inc.) was dissolved 100 mg/ml in DMSO, and then further diluted to a 10 mg/ml stock solution in 10% DMSO in deionized water. This stock solution was further diluted to appropriate working solutions in 1% DMSO prior to i.p. administration. Drug treatment in the L1210 leukemia model was initiated on Day 1, which was 1 day after L1210 cell implantation. Drug treatments in the remaining models (B16F10 murine melanoma, H460 human lung carcinoma, and WiDr human colon carcinoma) were initiated on Day 3. In the B16F10 murine melanoma model, the control mice received vehicle alone (1% DMSO in PBS), and the treatment mice received either 200 mg/kg cyclophosphamide (Sigma-Aldrich, Inc.) or Cloretazine (10, 30, 60 mg/kg) once a week for 3 weeks. There were ten animals per treatment group. In the H460 lung carcinoma experiment, there were 13 animals in the Cloretazine group, 12 animals in the cyclophosphamide group and in the control group. The control mice received vehicle alone, and treated mice received either 200 mg/kg cyclophosphamide or Cloretazine once a week for 5 weeks. Cloretazine doses were 60 and 100 mg/kg, then escalated to 100 and 150 mg/kg for the last two doses, respectively. In the WiDr colon carcinoma experiment, there were 11 animals in each group. In the L1210 leukemia experiments, control animals received vehicle alone every other day for a total of five doses. The treated animals received a single dose of Cloretazine (5 or 10 mg/kg) on Day 1, either alone or in combination with fludarabine (Berlex, Inc.) at a dose of 70 mg/kg every other day for a total of five doses. All control and drug treatments were administered i.p. in a volume of 0.2 ml. Results were analyzed by a Kaplan–Meier plot.

Tumor measurements and statistical analysis

The length (L), width (W), and height (H) of tumors were measured using electronic calipers. Tumor volumes were calculated using the following formula: tumor volume = $(L \times W \times H)/2$. The mean tumor volume, and standard deviation were determined for each animal group. The statistical significance of the differences between treatments was determined using the Student's t test (two-tailed). All experiments were repeated at least two times.

Results

The antitumor activity of Cloretazine relative to controls or cyclophosphamide was analyzed in three tumor models: B16F10 murine melanoma in C57BL/6 mice, H460 human lung, and WiDr human colon carcinoma in nude mice. Cloretazine significantly inhibited tumor growth in all three tumor models. The inhibitory effects were dose dependent, and the mean tumor growth inhibition ranged from 45 to 87% relative to vehicle alone (Figs. 1, 2, 3).

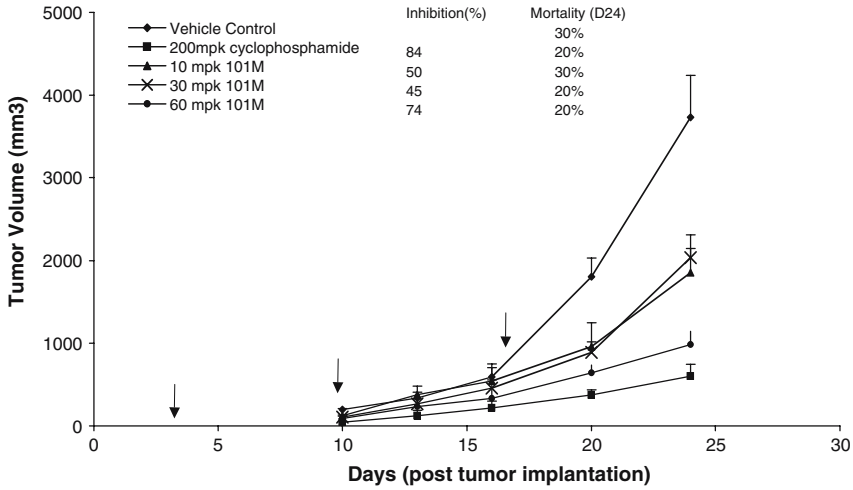

Fig. 1 Tumor growth inhibition of B16F10 melanoma in C57BL/6 mice by Cloretazine. There were ten animals per treatment group. Treatment was initiated on Day 3 post-tumor implantation and was given i.p. once per week for 3 weeks with 10, 30, 60 mg/kg Cloretazine, 200 mg/kg cyclophosphamide or vehicle alone. *Arrows* indicate the times of dosing

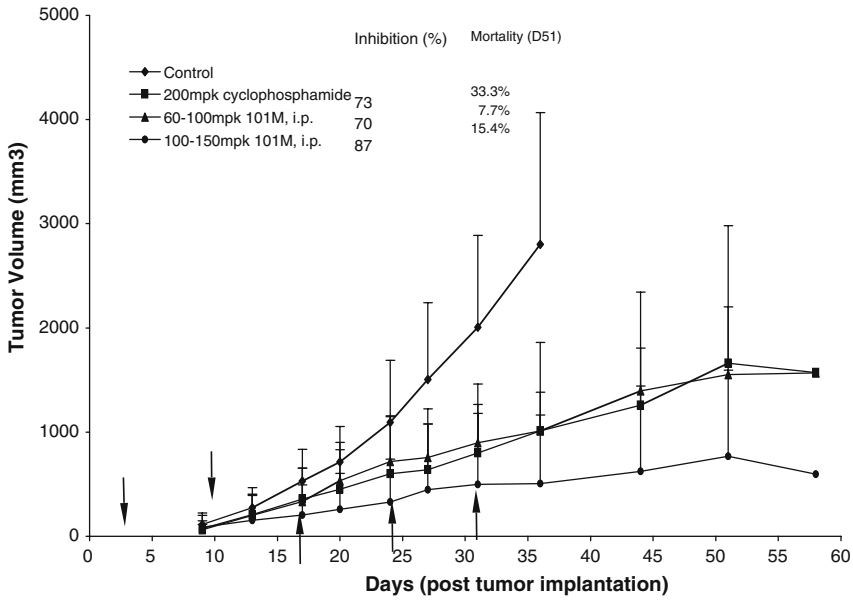

Fig. 2 Tumor growth inhibition of H460 human lung carcinoma in Nude CD1 mice by Cloretazine. There were 13 animals in Cloretazine group, 12 animals in the cyclophosphamide group and in control group. On Day 3 post-tumor implantation, the mice received an i.p. injection of 200 mg/kg of cyclophosphamide, Cloretazine (initially 60 or 100 mg/kg), or vehicle. Treatments were given weekly for 5 weeks. Cloretazine doses were escalated to 100 mg/kg (from 60 mg/kg group) and 150 mg/kg (from 100 mg/kg group) for week 4 and 5. *Arrows* indicate the times of dosing

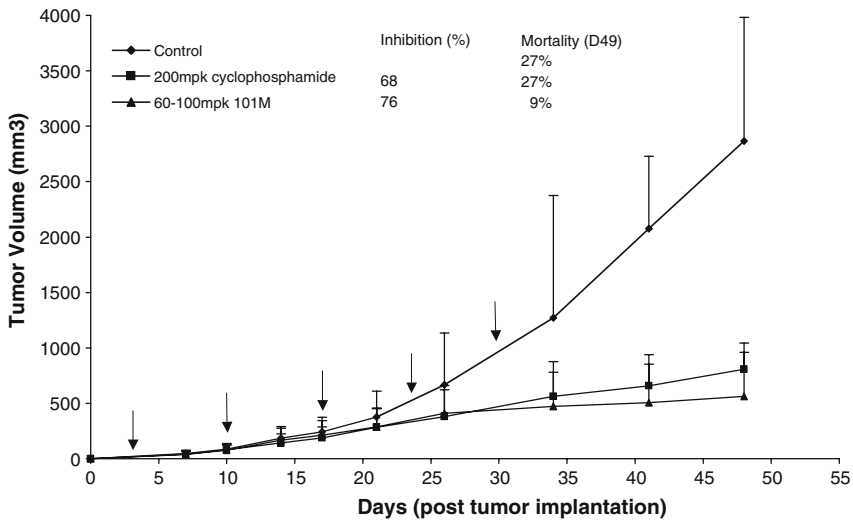

Fig. 3 Tumor growth inhibition of WiDr human colon carcinoma in Nude CD1 mice by Cloretazine. There were 11 animals per group. On Day 3 post-tumor implantation, the mice received an i.p. injection of 200 mg/kg of cyclophosphamide, Cloretazine (initially 60 mg/kg) or vehicle. Treatments were given weekly for 5 weeks. Cloretazine dose was escalated to 100 mg/kg for week 4 and 5. *Arrows* indicate the times of dosing

C57BL/6 mice bearing the B16F10 melanoma were treated once per week for 3 weeks with 10, 30, or 60 mg/kg Cloretazine, 200 mg/kg cyclophosphamide, or vehicle alone (Fig. 1). Significant inhibition of tumor growth was found with all doses of Cloretazine as determined by the Student's *t* test ($P < 0.05$). A dose of 60 mg/kg was found to inhibit tumor growth most effectively (74% mean inhibition, Fig. 1), while having a minimal effect on mean body weight (data not shown). Mortality rates were between 20 and 30% and were similar across all treatment groups.

Cloretazine treatment was evaluated next in nude CD1 mice bearing H460 human lung carcinoma cells. Mice received either cyclophosphamide 200 mg/kg, Cloretazine 60 and 100 mg/kg, initially or vehicle alone weekly for 5 weeks. Cloretazine doses were escalated to 100 and 150 mg/kg for week 4 and 5, since the initial doses yielded very little body weight loss. Cloretazine significantly inhibited tumor growth at both the low- and high-dose schedules in the H460 xenograft model ($P = 0.003$ and $P = 0.002$, respectively) (Fig. 2). Tumor growth inhibition was dramatic: a mean growth inhibition of 87% was observed in the mice treated with high-dose Cloretazine. Although the tumor growth inhibition was not significantly different ($P = 0.13$) (Fig. 2), the mortality rate of the mice treated with Cloretazine was less than that of the mice treated with cyclophosphamide. In animals bearing the H460 tumor xenograft, the mean body weight loss of the animals receiving the higher dose of Cloretazine was only 0.5% after treatment. All control animals had relatively large tumors, necessitating sacrifice on Day 36. A 15.4% mortality rate (2 of 13 mice) was observed in the high-dose Cloretazine-treated group by Day 51, and a 33.3% mortality rate (4 of 12 mice) was observed in the cyclophosphamide-treated group. Mice were treated with a low dose of Cloretazine experienced a mortality rate of only 7.7% by Day 51. During the study, autopsy of the mice revealed peritoneal metastasis adjacent to the tumor implantation site. Microscopic observation of blood smears from animals that received drug treatment indicated a loss of leukocytes relative to control animals.

Similar results were obtained in nude CD1 mice bearing the WiDr human colon carcinoma (Fig. 3). In the WiDr model, mean tumor growth inhibition in Cloretazine-treated mice was 76% (60–100 mg/kg Cloretazine). This growth inhibition, relative to the controls, was significant when analyzed by Student's *t* test ($P = 0.031$). Cloretazine treatment yielded a 9% mortality rate (1 of 11 mice), compared to a 27% mortality rate (3 of 11 mice) in cyclophosphamide-treated mice (Fig. 3).

Combination therapies of Cloretazine plus fludarabine were also evaluated in BDF1 mice bearing the L1210 leukemia. The combination therapy consisting of a single i.p. dose of Cloretazine (10 mg/kg) on Day 1 and five doses of fludarabine (70 mg/kg) given on every other day from Day 1, yielded a 90% survival rate on Day 68 post-tumor implantation (Fig. 4). Since the mice receiving 10 mg/kg Cloretazine monotherapy had a survival rate of only 40%, the combination therapy yielded a significant improvement in survival ($P = 0.023$).

Discussion

Cloretazine has previously been shown to have broad-spectrum antineoplastic activity in various murine

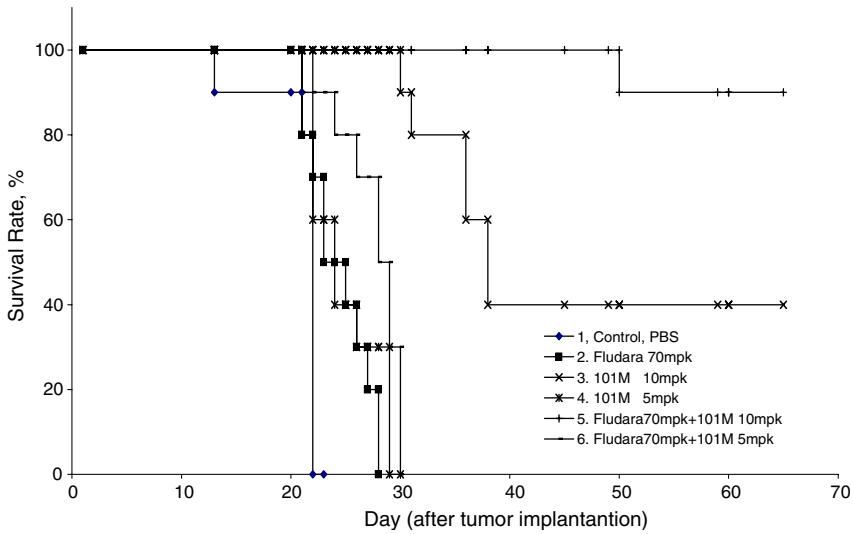

Fig. 4 Treatment of leukemia with Cloretazine and fludarabine. There were ten animals per group. The mice were inoculated i.p. with 1×10^6 L1210 leukemia cells and treated with Cloretazine, fludarabine, or combination therapy, as indicated. Treatments were given 1 day after tumor inoculation and repeated every 2 days for a total of five treatment

syngeneic and xenograft tumors [7]. Furthermore, not only was Cloretazine treatment effective in solid and hematological tumor models, but Cloretazine treatment in mice bearing L1210 leukemia or C26 colon carcinoma indicated an improved therapeutic index compared to the chemotherapeutic agent, BCNU [7]. In this study, we extend these results by showing that Cloretazine treatment of mice bearing B16F10 murine melanoma, H460 human lung carcinoma, or WiDr human colon carcinoma xenografts is equivalent to the clinically validated chemotherapeutic agent, cyclophosphamide. Furthermore, in the WiDr and H460 xenograft models, we demonstrate that Cloretazine has less toxicity than cyclophosphamide while maintaining similar efficacy.

In the H460 human lung carcinoma xenograft, the lower dose of Cloretazine and cyclophosphamide produced very similar tumor growth inhibition, but by Day 51, Cloretazine proved less toxic, as evidenced by a much lower mortality rate (33.3 vs. 7.7%). Interestingly, despite the drug-related toxicity of Cloretazine, the treatment still resulted in very little body weight loss, suggesting that the treatment might be reasonably well-tolerated by the surviving mice.

Similarly, in the WiDr human colon carcinoma xenograft model, marginally better tumor growth inhibition was observed in mice treated with the low dose of Cloretazine (76% inhibition) compared to the mice treated with cyclophosphamide (68% inhibition). Moreover, Cloretazine treatment resulted in a much lower mortality rate (9% for Cloretazine versus 27% for cyclophosphamide). These monotherapy experiments indicated an improved safety profile for Cloretazine when compared to cyclophosphamide, and suggest a greater therapeutic applicability for this agent.

We expected the combination of Cloretazine plus fludarabine to be particularly beneficial, since Cloretazine demonstrates similar efficacy and less toxicity compared to two different alkylating agents (this study and reference no. 7). In addition, fludarabine exhibits clinical efficacy when administered with cyclophosphamide to lymphoma patients [23]. In this study we observed dramatic and significant increases in long-term survival in L1210 leukemic mice treated with 10 mg Cloretazine plus 70 mg fludarabine. The long-term survival after treatment with this combination of agents was 90%, as compared to a 40% long-term survival rate for 10 mg Cloretazine alone, and a 0% long-term survival rate for fludarabine alone. Our results suggest that the treatment of leukemias with a combination of Cloretazine and fludarabine warrants further investigation (Table 1).

Table 1 Cloretazine plus fludarabine combination therapy in L1210 tumor-bearing BDF1 mice

Group	% Long-term survival (D68)	*P* value*
Control, PBS	0	
Fludarabine 70 mg/kg	0	
101 M 10 mg/kg	40	
101 M 5 mg/kg	0	
Fludarabine 70 mg/kg + 101 M 10 mg/kg	90	0.02324
Fludarabine 70 mg/kg + 101 M 5 mg/kg	0	

**P* value indicates a comparison between the 10 mg/kg Cloretazine treatment with and the control fludarabine

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